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Heterologous PrP Molecules Interfere with Accumulation of Protease-Resistant PrP in Scrapie-Infected Murine Neuroblastoma Cells

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Mutations within a host cellular protein, PrP, have been associated with disease in the transmissible spongiform encephalopathies. Murine neuroblastoma cells persistently infected with mouse scrapie accumulate protease-resistant PrP (PrP-res), the abnormal form of PrP associated with disease in the transmissible spongiform encephalopathies. These cells provide a controlled system in which to study the molecular interactions which are important in the formation of PrP-res. We have expressed recombinant PrP molecules in mouse scrapie-infected murine neuroblastoma cells and assayed the effect of these heterologous PrP genes on the formation and accumulation of PrP-res. The results demonstrate that expression of heterologous PrP molecules which differ from the endogenous PrP by as little as one amino acid can profoundly interfere with the overall accumulation of PrP-res. The data suggest that precise interactions between homologous PrP molecules are important in PrP-res accumulation and that heterologous PrP molecules can block these interactions.

Scrapie is a transmissible spongiform encephalopathy (TSE) which occurs naturally in sheep. A major characteristic of this neurodegenerative brain disease is the accumulation in the brain and other tissues of an abnormal form of a host protein, PrP (3, 17). The normal protease-sensitive form of PrP (PrP-sen) is the precursor to the abnormal, protease-resistant form of the molecule (PrP-res) (12), but the process by which PrP-sen is converted into PrP-res is unknown. Although the association of PrP-res with the TSE infectious agent remains controversial, PrP-res appears to be important in disease pathogenesis (1, 26, 32, 41). Moreover, genetically engineered mice in which the PrP gene is not expressed do not exhibit any disease symptoms following infection with mouse scrapie (6). Transgenic mice expressing high levels of hamster PrP (HaPrP) are susceptible to hamster-adapted scrapie strains, indicating a possible role for the PrP molecule in the species tropism of scrapie (34, 42). Furthermore, variations in scrapie incubation times in mice have been genetically linked to different PrP alleles (5, 8, 23, 38, 45). In human TSEs, mutations at single amino acid residues in the human PrP gene are associated with disease in Gerstmann-Straussler-Scheinker syndrome and familial forms of Creutzfeldt-Jakob disease (18, 19, 21, 22, 30). All of these studies suggest that differences in the protein sequence of PrP can significantly alter the course of disease in the TSEs.

Previous experiments using scrapie-infected cell lines expressing chimeric mouse-hamster PrP indicated that the PrP amino acid sequence could influence the incorporation of chimeric PrP molecules into PrP-res (44). In the present study, using similar recombinant PrP molecules, we found that expression of certain heterologous PrP molecules in mouse scrapie-infected cells could actually interfere with the overall generation and/or accumulation of PrP-res.

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MATERIALS AND METHODS

Cells. The uninfected mouse neuroblastoma cell line (MNB) and the mouse scrapie-infected neuroblastoma cell line (Sc^+-MNB) have been described previously (35, 37).

Antibodies. The anti-Friend murine leukemia virus (MuLV) mouse monoclonal antibody 720 and the anti-MuLV *gag* mouse monoclonal antibody R188 have been described previously (14, 39). Antibody R188 reacts with the murine spleen focus-forming retroviral *gag* gene expressed by the pSFF vector used in this study. The anti-HaPrP mouse monoclonal antibody 3F4 (25) recognizes an epitope within HaPrP which includes two methionine residues at positions 109 and 112 in the HaPrP sequence (4). All clones except the mouse PrP (MoPrP) clone contained the 3F4 epitope of HaPrP and reacted with the 3F4 mouse monoclonal antibody in a live-cell immunofluorescence assay (summarized in Fig. 1). Rabbit polyclonal antibodies R.27 and R.34 were raised to a PrP peptide encompassing amino acid residues 89 to 103 and have been described previously (13).

Clones. The cloning of HaPrP, MoPrP-M108/M111 (MoPrP containing methionines at positions 108 and 111), and FE (Friend MuLV envelope) into the pSFF vector have been published previously (15). MoPrP-L108/M111 (MoPrP containing leucine at position 108 and methionine at position 111; Fig. 1) was generated in wild-type MoPrP by PCR mutagenesis using the primer 5′-AGCGCCGGCCATATGCTTGAG GTT-3′, which changed the valine at position 111 to a methionine.

Clone SP40 was generated by recombining the 5′ end of the HaPrP gene and the 3′ end of the MoPrP gene, using a unique *Nae*I site in the HaPrP gene (Fig. 1). This site had been introduced into MoPrP-M108/M111 as described previously (15).

The clone designated MoPrP was identical in sequence to

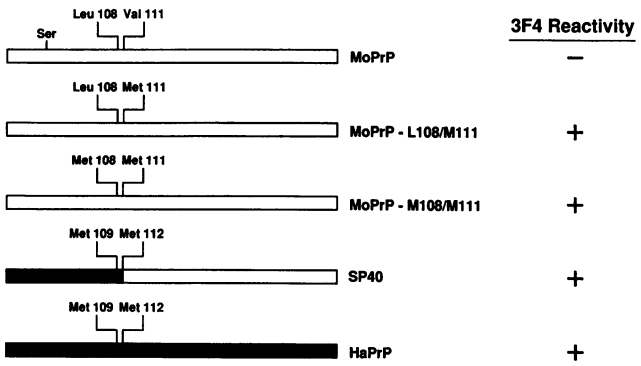

FIG. 1. Mouse and hamster recombinant PrPs. Protein sequence derived from MoPrP is shown by open bars, while that derived from HaPrP is shown by solid bars. The two amino acid positions which are involved in 3F4 reactivity are shown for each clone. The clone labeled MoPrP is PrP-a (31) but has a threonine-to-serine change at amino acid 55. None of the other clones have this change. The HaPrP is from wild-type Syrian hamsters (33). MoPrP and MoPrP-M108/M111 are identical except for the two amino acid changes within the 3F4 epitope and the serine at position 55. The 3F4 reactivity of each clone by live-cell immunofluorescence assay is shown on the right. The numbers assigned to the amino acid residues reflect the fact that HaPrP contains one extra amino acid compared with MoPrP (31).

the endogenous PrP gene in MNB cells except for a threonine-to-serine change at position 55. This change was introduced by PCR mutagenesis of wild-type MoPrP by using the primer 5′-TACCCACCTCAGGGTGGATCCTGGGGGCAG-3′. This mutation produced a unique *Bam*HI restriction endonuclease site in MoPrP. This is the only clone used in these studies which had this amino acid change.

Infection and analysis of Sc^+-MNB expressing recombinant PrPs. Transfection of all clones into retroviral packaging cell lines and production of infectious retroviral supernatants have been described before (15, 40). Supernatants containing infectious retroviral vectors encoding the recombinant PrPs were used to infect Sc^+-MNB cells. Expression of the recombinant vector was assayed by immunofluorescence using the 3F4 anti-PrP monoclonal antibody, the anti-FE monoclonal antibody 720, or the anti-MuLV *gag* monoclonal antibody R188. Following infection, Sc^+-MNB cells expressing heterologous PrP molecules were either cloned by limiting dilution or expanded to 75-cm^2 tissue culture flasks and grown to confluence. Single-cell clones were assayed for the expression of 3F4 epitope-containing PrP by live-cell immunofluorescence as previously described (11). Cell cultures expanded into 75-cm^2 flasks were assayed for PrP-res by immunoblotting using the alkaline phosphatase-based ProtoBlot immunoscreening system (Promega) as previously described (11) or the enhanced chemiluminescence reagent system (ECL; Amersham) according to the manufacturer's instructions.

Infectivity assay. Parental Sc^+-MNB cultures which expressed no construct, MoPrP-M108/M111, SP40, or HaPrP were grown to confluence in a 75-cm^2 tissue culture flask, and a cell suspension was prepared as published previously (35). Weanling Rocky Mountain Laboratory mice or 3-week-old Syrian hamsters (Charles River Laboratories, St. Louis, Mo.) were inoculated intracranially with 50 μl of a 1.0-ml cell suspension. All animals were periodically observed for clinical signs of scrapie.

Radioimmunoprecipitation of PrP-sen and PrP-res. Radioimmunoprecipitation of PrP-sen and PrP-res from ^{35}S-labeled Sc^+-MNB cell lysates was done as detailed elsewhere (12). Briefly, 25-cm^2 tissue culture flasks of confluent Sc^+-MNB cells were preincubated for 1 h in cysteine-methionine-free medium. Cultures were labeled for 2 h with Trans^{35}S-label (methionine plus cysteine; NEN), followed by a 24-h chase in normal tissue culture medium in the case of PrP-res. To label PrP-sen, cells were labeled for 2 h and harvested with no chase. Cells were lysed, and PrP-res was immunoprecipitated from proteinase K-treated cell lysates by using the anti-PrP polyclonal antibody R.34. PrP-sen was immunoprecipitated from cell lysates which had not been proteinase K treated.

TABLE 1. Attempted single-cell cloning of MNB cells expressing both PrP-res and 3F4-reactive recombinant PrP

Construct	Expt	% $3F4^+$ cells in parental culture[a]	No. of PrP-res$^+$ clones/total[b]	
			$3F4^{+c}$	$3F4^{-d}$
MoPrP-M108/M111	1	50	0/14	ND
	2	75	0/15	2/6
HaPrP	1	90	0/12	ND
	2	50	0/9	ND
	3	50	0/9	4/4
	4	40	4/10	ND
SP40	1	40	0/6	3/23

[a] Percentage of Sc^+-MNB cells positive for 3F4-reactive recombinant PrP as assayed by live-cell immunofluorescence in parental Sc^+-MNB cultures.
[b] Number of single-cell clones positive for PrP-res as assayed by immunoblotting over total number of clones.
[c] Number of single-cell clones positive for PrP molecules expressing the 3F4 epitope as assayed by live-cell immunofluorescence.
[d] Number of single-cell clones negative for PrP molecules expressing the 3F4 epitope as assayed by live-cell immunofluorescence. ND, not done.

RESULTS

Attempts to clone Sc^+-MNB cells which express both heterologous PrP molecules and PrP-res. We attempted to clone Sc^+-MNB clones which expressed one of three different PrP molecules in order to analyze the effect of the expression of these foreign PrP molecules in these cells. Expression of the recombinant PrP molecule was assayed by using monoclonal antibody 3F4, which recognizes only PrP derived from the recombinant PrP genes and not PrP derived from the endogenous MoPrP gene expressed in these cells. Despite the expression of the recombinant PrPs on the surface of a large number of cells, it was difficult to isolate single-cell clones which stably expressed both the exogenous PrP molecule and PrP-res (Table 1). In the four clones that were both HaPrP and PrP-res positive, the expression of both was unusually weak (data not shown). This finding suggested that expression of these recombinant PrP molecules in Sc^+-MNB cells was incompatible with PrP-res synthesis. To investigate this possibility further, we tested the more immediate effects of heterologous PrP expression on PrP-res accumulation in the parental Sc^+-MNB cultures which expressed these recombinant PrPs.

Interference of recombinant PrP with PrP-res accumulation. Sc^+-MNB cells usually synthesize PrP-res from an endogenous, wild-type MoPrP gene which does not express the 3F4 antibody epitope. The amount of PrP-res present in uncloned Sc^+-MNB cultures which expressed the MoPrP-M108/M111 construct (Fig. 1) was assayed by Western blotting (immunoblotting) with polyclonal rabbit antiserum R.27. This antiserum should detect PrP-res derived from both the endogenous and recombinant PrP molecules, since the peptide sequence to

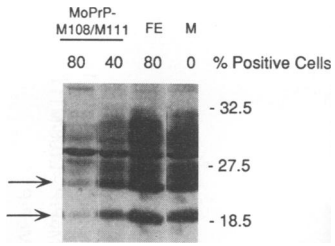

FIG. 2. Endogenous PrP-res production in Sc^+-MNB cells is dependent on the number of cells expressing the MoPrP-M108/M111 construct. Western blot analysis of proteinase K-treated cell lysates from Sc^+-MNB cells with a high (80%) or low (40%) number of cells expressing the MoPrP-M108/M111 construct is shown. The percentage of cells expressing the indicated construct was assayed by immunofluorescence using either the anti-PrP monoclonal antibody 3F4 or the anti-FE monoclonal antibody 720 (FE lane only) and is indicated above each lane. Equivalent numbers of cells were loaded in each lane. Blots were developed by using the anti-PrP rabbit polyclonal antibody R.27, an alkaline phosphatase-conjugated goat anti-rabbit antiserum, and the alkaline phosphatase development procedure as described previously (11). The results were reproducible over several experiments. Arrows at the left indicate the major PrP-res bands, and positions of molecular weight markers (in kilodaltons) are indicated on the right. The band at 28 kDa is not PrP specific. M, no construct.

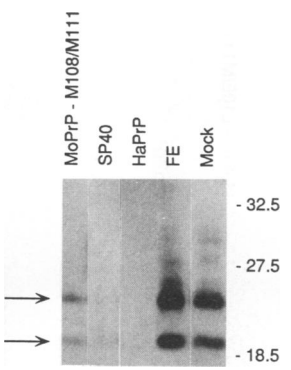

FIG. 3. Recombinant PrP molecules interfere with endogenous PrP-res accumulation in Sc^+-MNB cells. Western blot of proteinase K-treated cell lysates from Sc^+-MNB cells expressing different recombinant PrPs. Identical cell equivalents were loaded in each lane. Blots were developed with the anti-PrP rabbit polyclonal antibody R.27 as in Fig. 2 except that development was with the ECL system. Development with the ECL system consistently demonstrated greater sensitivity and lower backgrounds than the color development system used for Fig. 2. The results were reproducible over several experiments. The two major PrP-res bands are indicated by the arrows on the left, and positions of molecular weight markers (in kilodaltons) are shown on the right. Mock, no construct.

which it was made is common to both mouse and hamster PrP (13). Sc^+-MNB cultures in which 80% of the cells expressed the MoPrP-M108/M111 recombinant demonstrated a greater reduction in total PrP-res than cultures in which only 40% of the cells expressed this construct compared with Sc^+-MNB cultures which expressed either Friend envelope or no construct (Fig. 2). Thus, the reduction in PrP-res in these cells correlated well with the number of cells which synthesized the 3F4-positive PrP molecules. The data suggested that the heterologous PrP molecules interfered with the accumulation of PrP-res in cells in which they were expressed, and we decided to investigate this possibility further.

Uncloned Sc^+-MNB cultures which expressed one of three different PrP molecules were assayed for expression of total PrP-res, using polyclonal rabbit antibody R.27. Cells which expressed MoPrP-M108/M111, SP40, or HaPrP showed a drastic reduction or elimination of immunoreactive PrP-res (Fig. 3). Samples in which PrP-res was separated from PrP-sen in the absence of proteinase K treatment by differential centrifugation alone showed similar results (data not shown). The decrease in PrP-res was not due to expression of any non-PrP genes contained within the pSFF vector because mock-infected Sc^+-MNB and Sc^+-MNB cultures which expressed the FE gene in the pSFF vector both produced high levels of PrP-res. MoPrP-M108/M111 differed from the wild-type MoPrP expressed in these cells by only two methionine residues at positions 108 and 111. The results suggested that the presence of the two methionines within the 3F4 epitope of the heterologous PrP (Fig. 1) led to the reduced accumulation of PrP-res in scrapie-infected cells.

Effect of methionine at position 111 on PrP-res accumulation. To determine the importance of the individual methionine residues in the accumulation of PrP-res in Sc^+-MNB cells, clone MoPrP-L108/M111 (Fig. 1) was tested for its ability to affect PrP-res synthesis. Control Sc^+-MNB cultures which did not express any exogenous PrP molecules produced high levels of protease-resistant PrP (Fig. 4A, Mock). When the cells expressed an exogenous MoPrP gene which was not mutated at the 3F4 epitope, an even higher level of PrP-res, presumably due to the increased amount of MoPrP-sen synthesized, was detected (Fig. 4A, MoPrP). By contrast, cultures which expressed clone MoPrP-L108/M111, which differed from the endogenous MoPrP only at methionine 111, demonstrated the same dramatic reduction in PrP-res seen with the MoPrP-M108/M111 construct (Fig. 4A). Sc^+-MNB cells which expressed either of these constructs showed ~85% reductions in total PrP-res compared with control samples which expressed no construct (Fig. 4B). These results measured the amount of PrP-res accumulated in these cells over the course of 5 to 7 days. We also measured the amount of PrP-res which accumulated in these cells over 24 h by pulse-chase labeling and immunoprecipitation. Similar results were seen (Fig. 4C). Therefore, a single amino acid change from valine to methionine at position 111 within the exogenous PrP was sufficient to significantly reduce the accumulation of PrP-res in Sc^+-MNB cells.

Expression of PrP-sen in Sc^+-MNB cells expressing exogenous PrP molecules. We also assayed the level of PrP-sen expressed in these cells by using the anti-PrP polyclonal antibody R.34. The biosynthesis of PrP-sen in Sc^+-MNB cultures which expressed the PrP constructs was monitored by [^{35}S]methionine labeling and immunoprecipitation. The data showed that all of the cell cultures synthesized PrP-sen (Fig. 5). In fact, PrP-sen biosynthesis in Sc^+-MNB cells which expressed heterologous PrP molecules was higher than that in control cells or cells which expressed the FE gene. This was probably a result of the presence of extra copies of the PrP gene transferred into these cells in the expression vector.

These results did not exclude the possibility that the observed reduction in total PrP-res accumulation was due to the reduced expression of the endogenous MoPrP gene. To address this possibility, a [^{35}S]methionine-labeled lysate from Sc^+-MNB cells expressing exogenous PrP from clone MoPrP-M108/M111 was immunoprecipitated with monoclonal antibody 3F4 to remove exogenous PrP. After five cycles of immunoprecipitation with 3F4, endogenous MoPrP was found to be present at normal levels by immunoprecipitation with the

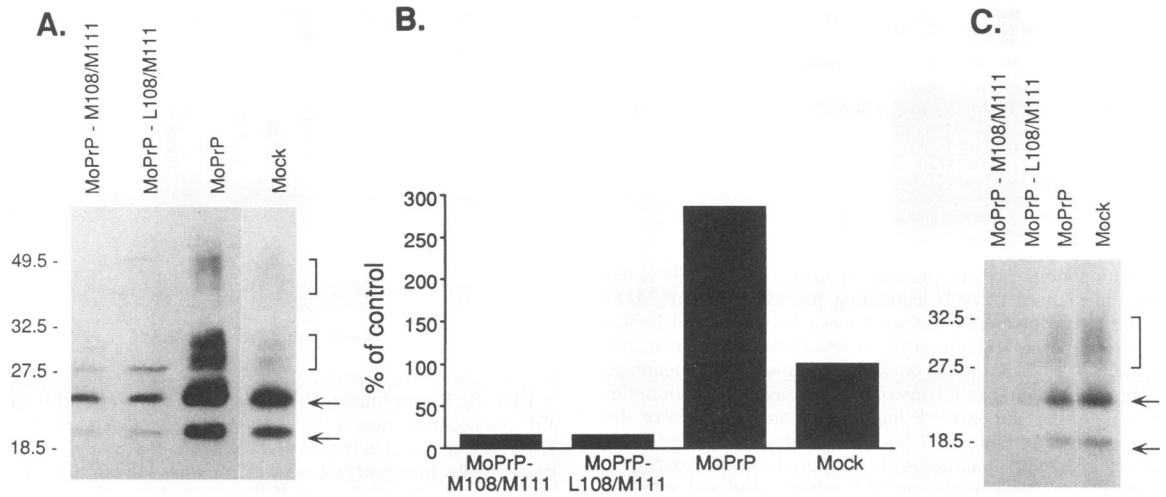

FIG. 4. Recombinant PrPs mutated at the 3F4 epitope interfere with production of endogenous PrP-res in Sc^+-MNB cells. (A) Western blot analysis of proteinase K-treated cell lysates from Sc^+-MNB cells expressing PrP clones mutated within the 3F4 epitope. Cell equivalents were comparable in each lane. The immunoblot was developed as described in the legend to Fig. 3, using the anti-PrP polyclonal antibody R.27 as the primary antibody. (B) Graphical representation of a densitometric scan of the immunoblot in panel A. (C) Immunoprecipitation of PrP-res from proteinase K-treated cell lysates of Sc^+-MNB cells expressing PrP clones mutated within the 3F4 epitope. PrP-res was immunoprecipitated with rabbit polyclonal antibody R.34. The arrows on the right indicate the two major PrP-res bands, and positions of molecular weight markers (in kilodaltons) are shown on the left. The bands present between 27.5 and to 32.5 kDa and between 40 and 50 kDa (brackets) are also PrP-res specific. The results were reproducible over several experiments. Mock, no construct.

polyclonal anti-PrP antiserum R.34 (Fig. 6). These results demonstrated that the level of expression of the endogenous MoPrP gene was not affected by the presence of an exogenous PrP molecule. Therefore, the reduction in PrP-res accumulation in these cells was not due to a reduction in the level of expression of the endogenous MoPrP gene.

Scrapie infectivity in Sc^+-MNB cells expressing heterologous PrP molecules. To determine if the reduction in PrP-res accumulation affected the scrapie infectivity in Sc^+-MNB cells, cell lysates from Sc^+-MNB cells expressing MoPrP-M108/M111, SP40, and HaPrP were used to infect mice by intracranial inoculation. Despite the significant reduction in the amount of accumulated PrP-res, all of the Sc^+-MNB cultures which expressed foreign PrP genes still contained mouse scrapie agent (Table 2). As reported in another study (10), this Sc^+-MNB cell line had a low level of scrapie infectivity, and the similar incubation times produced by all the cell lysate inocula indicated that all of the cultures had similar titers of agent (35). Quantitative comparisons between scrapie infectivity and PrP-res are difficult (36). The bioassay for scrapie infectivity is ~100- to 1,000-fold more sensitive than the detection of PrP-res by immunoblotting (34a), so it is not surprising that infectivity could still be detected even in the apparent absence of PrP-res.

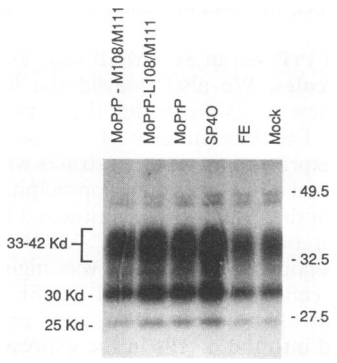

FIG. 5. Expression of 3F4-positive PrP clones in Sc^+-MNB cells does not adversely affect PrP-sen biosynthesis. PrP-sen was immunoprecipitated with the anti-PrP rabbit polyclonal antiserum R.34 from [^{35}S]methionine-labeled cell lysates of parental Sc^+-MNB cultures expressing different PrP recombinants. The data were reproducible over several experiments. The bands corresponding to PrP-sen and their molecular masses are shown on the left, and positions of molecular weight markers (in kilodaltons) are shown on the right. Mock, no construct.

Mouse and hamster PrP differ by only 14 amino acids (31), which may account for the species specificity of the scrapie agent. Results of recent experiments with transgenic mice which expressed high levels of recombinant PrP genes have suggested that PrP may play a role in the tropism of the scrapie agent (34, 42, 43). To determine if any hamster-tropic scrapie agent had been generated in Sc^+-MNB cells which expressed recombinant PrPs, hamsters were also infected by intracranial inoculation. None of the cultures contained hamster scrapie infectivity, even though 3F4-positive recombinant PrP-res molecules derived from some of the constructs were detected (Table 2). Similar results have been reported previously (44). Therefore, the presence of hamster-mouse recombinant PrP-res molecules derived from hamster-mouse recombinant PrP genes did not alter the tropism of the scrapie agent in Sc^+-MNB cells.

DISCUSSION

We have used Sc^+-MNB cells to express heterologous recombinant PrP molecules which differ from the endogenous MoPrP in these cells. We have shown that HaPrP, MoPrP containing the hamster 3F4 epitope (MoPrP-M108/M111), and MoPrP-L108/M111 strongly interfere with PrP-res accumulation. In contrast, Scott et al. (44) noted interference only with

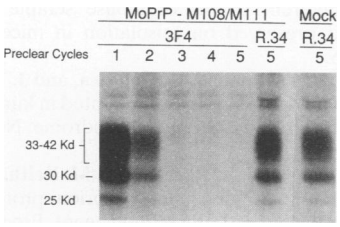

FIG. 6. Expression of the 3F4-positive MoPrP-M108/M111 clone in Sc^+-MNB cells does not affect expression of the endogenous MoPrP-sen gene. PrP-sen was immunoprecipitated from [^{35}S]methionine-labeled lysates of Sc^+-MNB cells which expressed the MoPrP-M108/M111 construct or Sc^+-MNB cells which expressed no construct (Mock), using monoclonal antibody 3F4 in five consecutive cycles of immunoprecipitation (preclear cycles 1 to 5, 3F4). At preclear cycle 5, the lysate was divided in half and immunoprecipitated with either 3F4 or R.34 as shown. Mock cells did not express any 3F4-positive PrP-sen molecules, and only the final round of immunoprecipitation with the anti-PrP polyclonal antibody R.34 is shown. The bands corresponding to PrP-sen and their molecular masses are shown on the left.

HaPrP but not with MoPrP-M108/M111. The reason for this discrepancy is unknown. However, the level of exogenous PrP expression can vary depending on the construct used to drive expression and the percentage of cells expressing the exogenous PrP gene (Fig. 2). Either of these two variables might account for the difference between the results of Scott et al. (44) and those presented here.

Allelic variation at amino acid residue 108 within the region defined as the 3F4 epitope has previously been correlated with altered scrapie incubation times in mice (45). The mechanism of this effect is not known, but the results presented above suggest that the amino acid substitution at position 108 in PrP may decrease the amount of PrP-res accumulated, which in turn might prolong the course of disease. Although it has been hypothesized from previous in vivo studies that interactions between nonhomologous PrP proteins may adversely affect the disease process (7, 34, 43), our studies are the first to demonstrate directly that heterologous PrP molecules which differ by as little as one amino acid can interfere with the accumulation of PrP-res.

When mouse scrapie agent is inoculated into transgenic mice expressing high levels of HaPrP, disease incubation times are significantly longer than in nontransgenic mice (34). Our data suggest that HaPrP can significantly interfere with the production of PrP-res in mouse tissue culture cells infected with mouse scrapie. It is possible that a similar reduction in total PrP-res accumulation, which leads to a slower accumulation of PrP-res and increased incubation times, is occurring in the HaPrP transgenic mice. Interference of PrP-res accumulation by heterologous PrP molecules might also provide an explanation for the initial long incubation times observed when a strain of scrapie agent is passaged into a new host (27–29).

The interference of heterologous PrP molecules with PrP-res accumulation might be the result of a disruption of the process by which PrP-sen is converted into PrP-res. For example, interactions between dissimilar PrP-res and PrP-sen molecules might slow the aggregation and accumulation of PrP-res by interfering with interactions between PrP monomers (2, 20, 34). Consistent with this hypothesis and in agreement with previous studies (44), both the MoPrP-M108/M111 and SP40 recombinant PrPs were incorporated to some extent into PrP-res (Table 2), even though they still interfered with PrP-res accumulation (Fig. 3). Similarly, incorporation of nonhomologous PrP molecules into PrP-res aggregates might lead to a destabilization of the aggregates (24), and some evidence for this has recently been found (16). Exogenous PrP molecules might also inhibit the interaction of the endogenous PrP with cellular ligands such as proteoglycans, an interaction which is thought to be important in the conversion of PrP-sen into PrP-res (9).

TABLE 2. Scrapie infectivity of Sc^+-MNB cells expressing recombinant PrPs

Construct[a]	% of control[b]	3F4+ PrP-res[c]	No. dead of scrapie/no. infected	
			Mice[d]	Hamsters[e]
None	100	−	21/21 (244 ± 17)	NT
MoPrP-M108/M111	15	+	8/8 (254 ± 31)	0/6
SP40	15	+	7/7 (222 ± 13)	0/6
HaPrP	0	−	8/8 (254 ± 13)	0/6

[a] Sc^+-MNB cells expressing a recombinant PrP construct.
[b] PrP-res accumulation as assayed by immunoblotting, expressed as a percentage of the total PrP-res in Sc^+-MNB cells.
[c] Accumulation of 3F4-positive PrP-res as assayed by immunoblotting.
[d] The average time to death (days) ± standard deviation is shown in parentheses.
[e] Incubation time was >365 days. NT, not tested.

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